Original

Isoelectric Points Estimation of Proteins by Electroosmotic Flow: pH Relationship Using Physically Adsorbed Proteins on Silica Gel

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Abstract

A surface modification of the silica gel in a mini-packed polypropylene tube had been developed by physically adsorption of proteins. Several proteins (bovine serum albumin, pepsin, globulin, β-casein) had been attempted to physically adsorb on the silica gel in a mini-packed cartridge under simple conditions. The proteins were successfully adsorbed on the silica gel, thus, minimizing the effect of the silanol groups. The protein isoelectric point values (pI) might be determined from the pH of which the EOF is zero. The estimated pI values corresponded very well with the literature values. The switchable EOF property of the protein adsorbed phases are fruitful for the manipulation of the magnitude and direction of EOF by selecting pH of buffer solutions and proper adsorbed protein phases. Four replicate different mini-packed cartridges were used to observe the EOF by using a simple homemade apparatus. The technique was easily to handle and did not take too much time consuming.

Keywords: adsorption, proteins, isoelectric points, silica gels, mini-packed cartridge

Introduction

Proteins are polyelectrolytes, and adsorption usually occurs because of the coulombic attractions between the negatively charged surfaces and the positive charges on the protein molecules. Adsorption at the surface was usually considered as a troublesome effect in CE, especially for the separations of proteins and peptides. The result is either tailing peaks or even complete adsorption of the protein to the surface. This can give rise to zone spreading, peak tailing, and adversely affect the repeatability of the separations of proteins and peptides [1-3]. However, the protein-bonded column was used to study the selectivity in liquid chromatography [4]. At present, the adsorption effect of proteins was used as an advantage for the separation of amino acids and was used as a chiral stationary phases for the enantiomers in OTCEC [5]. Proteins may strongly adsorbed on the silica gel. In this work, we utilize the adsorption of protein on silica gel in a mini-packed cartridge for the determination of its pI value.

Experimental

Instrumentation

A schematic diagram of an apparatus for measurements of the EOF velocity was nearly the same as our former report [6], with some adaptation as shown in Figure 1. The apparatus consisted of a 1-ml mini-packed polypropylene tube dipped in the buffer solution reservoir. The platinum wire electrodes were inserted to the tube and the medium reservoir. The cartridge was set vertically. As the level of the solution in a cartridge keep as the same as the level of the solution in reservoir, we could avoid almost completely the effect of gravity for the estimation of EOF. The voltage power supply (DC; type R2; Toyo Roshi Ltd., Tokyo, Japan) at a constant 200 volts was applied through the approximate 1-cm long packed bed. The total electric resistance is mostly reflected the resistance of its packed part. The approximate 200 volt/cm potential gradient was enough to generate the EOF of the protein adsorbed phases. The electric current of all media used were 1-3 mA.

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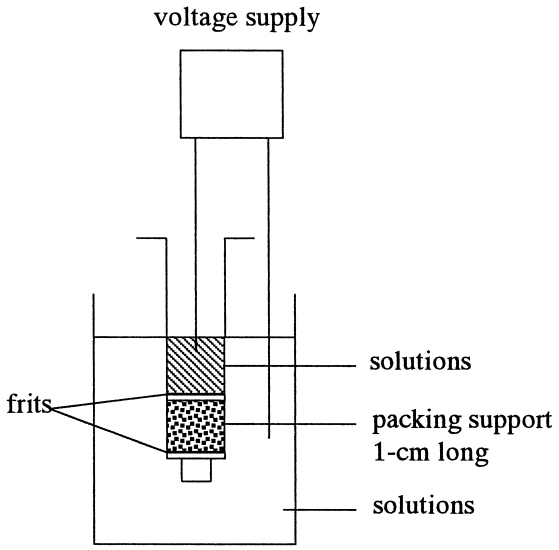

Figure 1. Schematic diagram of an apparatus for measurements of the EOF velocity of the protein adsorbed silica gel in a mini-packed polypropylene cartridge.

Cartridge packing, mobile phases and reagents

The packing supports were silica gel (LC-Sorb, 45 μm diameter, Lot no 401025, Chemco. Ltd., Osaka, Japan). The packing procedure was the same as in the former report [6]. Mobile phases used were the mixtures of the citric acid and disodium hydrogen phosphate of constant ionic strength (0.01) containing 1 mg/ml protein solution (0.4 mg/ml in case of β-casein). All aqueous buffer solutions were degassed by ultrasonic bath prior to use. The proteins used were bovine serum albumin (BSA), pepsin, globulin and β-casein. All reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Modification of the packed silica gel with proteins

Silica gel packed polypropylene tubes were activated by rinsing with 0.5 N sodium hydroxide and deionized water for 30 min and 15 min, respectively. The protein adsorption phase was generated by filling the mini-packed cartridge with the 10 mg/ml protein solution (4 mg/ml β-casein solution owing to low solubility) and keeping for 20 min. The solution was then flushed away by suction with syringe via luer tip end for 5 min. Cartridge was kept drying in an oven at constant 37 degree for 20 min. Then filling the mini-packed cartridge again with the protein solution, keeping for 20 min, flushing solution away for 5 min and drying cartridge at 37 degree for 20 min. The physically adsorbed proteins on silica gel packed cartridge was then used to observe the EOF at various pH buffer medium containing protein solutions.

Procedure

The protein coated adsorbents were equilibrated with the aqueous buffer solution for about 10 min prior to measure the EOF velocity. The solvents were moved by means of a syringe in the suction mode through the luer tip side in the voltage-off mode. EOF velocity was achieved by direct measuring the time used for the electroosmotic volume flow movement to the 1-2 mm distance in the upper open part of the cartridge under constant 200 volts application. All measurements were operated by using the homemade apparatus as shown in Figure 1. EOF velocity was calculated by using the same equation as described in our former report [6]. Each type of protein adsorbed phases, four replicate protein adsorbed silica gel cartridges were used for the measurement of EOF. As the reproducibility of EOF measurement by this technique was already well observed in our former report [6], each EOF velocity at each pH was the average value of four EOF measurements. In this work, the reproducibility of the adsorption of proteins on silica gel in a mini-packed cartridge was then observed as the %RSD of the EOF velocity of four different cartridges.

Results and Discussion

EOF as a function of pH

The plots of EOF as a function of pH for the protein adsorbed silica gel cartridge were shown in Figure 2 and 3. The effect of having both positive and negative charges on the protein adsorbed surface can be illustrated by the measurement of the EOF in the experiment. From Figure 2 and 3, it is apparent that both magnitude and direction of EOF change with the pH of the contacted medium. At the zero EOF, the phases exhibited no EOF, indicating that the surface of the phases had a net zero charges: this pH is referred to as the isoelectric point (pI) of the surface of the protein adsorbed phases. EOF direction is from the cathode to the anode if the adsorbed surface becomes positive charges as observed in the region below the surface isoelectric point. In the region of cathodic EOF above the surface pI, the proteins bear negative charges and the EOF direction is reversed. The magnitude of EOF increases with decreasing pH in the anodic EOF region and incresing pH in the cathodic EOF region, according to the amount of charged functional groups of coated protein stationary phase.

Estimation of isoelectric points of proteins

Isoelectric point is the pH at which the protein has zero net charge. In protein adsorbed silica gel, EOF is suppressed at about a surface isoelectric point. The zero EOF was estimated by curve-fitting the EOF velocity at different pH values. According to our former research [6], EOF velocity (v_{osm}) is related to the total surface charge density (σ_t), regardless of its source, as expressed as

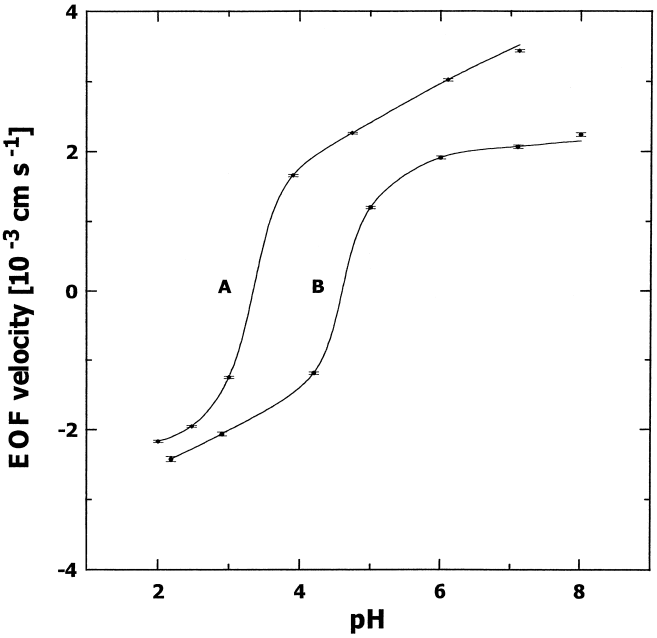

Figure 2. Plots of EOF velocity and pH for pepsin adsorbed silica gel phases (curve A) and for BSA adsorbed silica gel phases (curve B) at constant 200 applied voltage. The electrolyte solutions were citric acid-disodium hydrogen phosphate (ionic strength 0.01) containing 1 mg/ml protein solutions. The error bars showed the deviation of EOF velocity using four replicate cartridges.

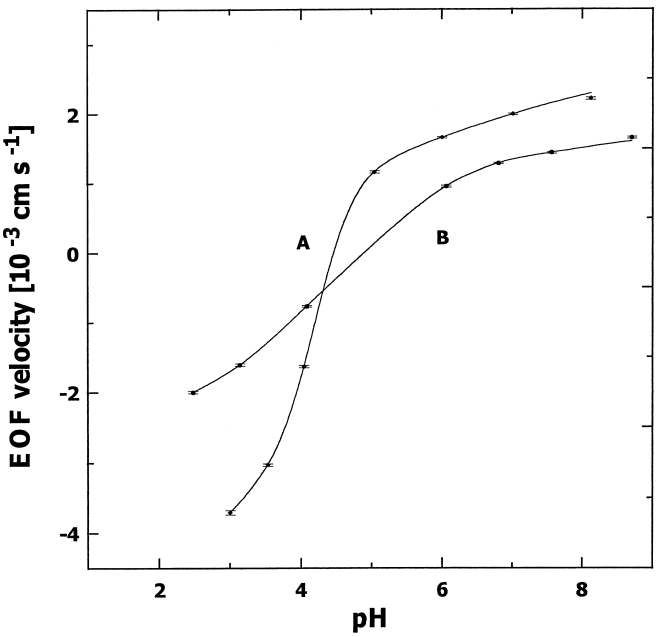

Figure 3. Plots of EOF velocity and pH for β-casein adsorbed silica gel phases (curve A) and for globulin adsorbed silica gel phases (curve B). The electrolyte solutions were the same as in Figure 2 except containing 0.4 mg/ml β-casein solutions. Other explanations were the same as in Figure 2.

$$C \sinh\nu_{osm} = \sigma_t = \sigma_n + \sum_{i=1}^{m} \sigma_{\text{functional group, i}}$$

$$= \sigma_n + \sum_{i=1}^{m} e\gamma_{\text{functional group, i}} K_{a,\text{functional group, i}} / (K_{a,\text{functional group, i}} + a_H)$$

C equals $\Delta E\ (\varepsilon/\eta)\ (\exp(-\kappa x))\ (2kT/e)\ (500\ \pi/\varepsilon RTc)^{-1/2}$, where ΔE, ε, η, κ, x, k, T, e, R and c are the applied voltage at unit length, permittivity, viscosity, inverse Debye length, radius of the counterion, Boltzmann constant, temperature, elementary charge, gas constant and concentration of ions, respectively. C is an approximate constant value under a given set of conditions if the applied voltage is constant. σ_n, $\sigma_{\text{functional group, i}}$, e, $\gamma_{\text{functional group, i}}$, $K_{a,\text{functional group, i}}$, a_H and m are naturally induced surface charge density, functional group surface charge density, the elementary charge, the number of surface functional groups per unit area, acid dissociation constant of the functional groups, activity of hydrogen ion and number of functional groups of proteins, respectively.

In case of protein adsorbed silica gel, the phases contained many different charged groups of protein. Therefore, the relationship between the EOF velocity and activity of hydrogen ion was in the rational form with multiparameters. The zero EOF could be estimated from the fitting procedure between the EOF velocity and activity of hydrogen ion. The reliability of the fitting procedure was indicated by the confidence coefficients [6], which were 99.5 % (p<0.005) in this paper. It confirmed very well with our former research [6].

From Figure 2 and 3, the titration curve shape of the plot of EOF and pH was achieved. The shape of the curves between the EOF velocity and pH resembles the titration curves with different slope and asymptote. Each curve in Figure 2 and 3 approached the asymptote at different pH. This might be the result of the different charged groups, compositions, and distribution of charged side chains of proteins. The pI values of the protein adsorbed on the silica gel might be estimated from the pH at zero EOF. In this report, the estimated pI values of the pepsin, BSA, β-casein and globulin were 3.2, 4.6, 4.5 and 5.0, respectively. These estimated pI values

Proteins	Experimental pI	Literature pI (7-9)
Pepsin	3.2	3.3 (7)
BSA	4.6	4.7 (8)
β-casein	4.5	4.5 (7,9)
Globulin	5.0	5.1 (7,8)

Table 1. Comparison of pI values of proteins from experiment and literature (from refs.7-9).

corresponded very well with the literature pI values [7-9] as compared in Table 1. From the experiment result, the outer part of the coated proteins on silica gel and the proteins in the solution give the same contribution to the pI estimation.

Adsorption of proteins on silica gel

Macromolecular substances often show greater tendencies to adsorb to any surface than do low-molecular weight compounds. The reason is that macromolecules especially proteins have many more binding sites and accordingly can be adsorbed by multipoint attachment [10, 11]. Furthermore, the binding sites can be stereoselective in nature, making them potentially useful in the separation of chiral molecules [12]. Therefore, we have been attempted to physically adsorb protein to silica gel in a mini-packed cartridge. The successful adsorption of proteins on silica gel could be illustrated by analyzing the relationship between EOF and pH of solutions. As in our former report [6], the in-situ pKa of silanol groups in a mini-packed cartridge were between 4.0 and 4.3. The effect of the existence of silanol groups could be seen from the inflection point of the plot between EOF and pHs at which the inflection point is nearly the same as the pKa value of the silanol groups. In this work, there were no inflection points at the pH around the pKa values of the silanol groups as shown in Figure 2-3 for all protein adsorbed silica gel phases. For different kinds of the proteins used for the physically adsorption on silica gel, the inflection points were around the surface pI values. These inflection points represented only for the pI values of the proteins. No other inflection points caused by the existence of silanol groups observed, thus, it implied the successful of physically coated proteins on silica gel in a mini-packed cartridge in this work and the amount of the adsorbed proteins is enough for the pI estimation.

According to our data concerning the adsorption effect of cetyl trimethy ammonium bromide (CTAB) on silica gel, there is an evidence of the incomplete adsorption effect during the running experiment if we use the running buffer containing no CTAB. Therefore, for more reliability and complete adsorption of proteins of all the running experiments, it is better to add the protein in the running electrolytes. The concentration we used in this experiment is the same as used in the former references experiments [10, 11].

The reproducibility of the EOF velocity of four different replicate physically protein adsorbed silica gel in a mini-packed cartridge was found to be within 1.6% RSD. This technique was successful diminish the existence effect of the free silanol groups of the silica gel.

Conclusion

The adsorption effect of the protein on surface, usually considered to be a troublesome effect, has been successfully utilized as a means of benefaction for preparation of the physically adsorbed proteins on silica gel in a mini-packed cartridge. The data presented in this report indicated the successful adsorption of proteins on silica gel. Effective protein stationary phases are obtained without interference from the existence of silanol groups. The adsorption protocols are promising and will become more useful on the separation of compounds; i.e., proteins, amino acids and chiral compounds. We believe this method minimizing the effect of the silanol groups. Therefore, the pI values could be obtained from the relationship between EOF and pH of the medium. This technique, a surface modification has been developed, which yields adsorbed protein phases with switchable EOF (anodal/cathodal). Because of the adsorption of proteins on the phases, the net charges of the phases can be varied from positive to negative by changing the pH of the contacted buffer, enabling the manipulation of EOF. This was the same as the description of the chemical modification of the inner surface of the fused silica capillary by adsorption with surface-active reagents (cetyltrimethylammonium bromide) [13, 14]. An additional aspect of such behavior is that, in principle, at any pH the magnitude and direction of EOF can be adjusted by selecting the proper adsorbed protein phases. Other physically adsorbed macromolecules and quaternary compounds on other phases are now under investigated.

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